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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant	:	Graff et al.	Examiner:	To Be Assigned
Serial. No	:	10/002,631	Group Art Unit:	To Be Assigned
Filed	:	October 31, 2001		
For	:	METHOD TO IDENTIFY SIGNAL SEQUENCES		

DECLARATION UNDER 37 C.F.R. §1.131

Professor Jonathon M. Graff residing at 3124 Milton Ave, Dallas, TX, 75205 and
Matthew R. Muenster residing at 2014 Royal Oaks Drive, Irving, TX 75060 declare as follows:

1. We are the applicants of the above-identified patent application and coinventors of the subject matter described and claimed therein.
2. Prior to March 9, 2001, we completed the invention described and claimed in the subject application, in this country. The following set of facts demonstrate conception and reduction to practice of the subject matter of the present invention prior to March 9, 2001, the filing date of Tan et al. (US2002/012755A1).
3. Prior to March 9, 2001, Matthew R. Muenster prepared a vector comprising an ampicillin resistance (AmpR) gene with the native signal sequence deleted as a backbone vector for cloning the vector of the present invention. A copy of the relevant notebook data pages is attached as Exhibit A. The dates from Exhibit A are redacted.
4. Prior to March 9, 2001, Matthew R. Muenster prepared a vector comprising genes encoding known secreted proteins, e.g. leptin, directionally cloned upstream of the AmpR gene. Matthew R. Muenster shows that host cells transformed with this vector survived on

selection media. Expression of a cDNA fragment encoding a protein comprising a signal sequence confers survival of the host cell on selection media. A copy of the relevant notebook data pages is attached as Exhibit B. The dates from Exhibit B are redacted.

5. Prior to March 9, 2001, Matthew R. Muenster confirmed the sequence of the vector comprising the leptin gene cloned upstream of the AmpR gene. A copy of the relevant notebook data pages is attached as Exhibit C. The dates from Exhibit C are redacted.

6. Each of the dates redacted from Exhibits A-C is prior to March 9, 2001.
Furthermore, each of the dates redacted from Exhibits A-C is prior to March 30, 2001.

7. We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



Jonathon M. GRAFF

Date: 7.1.03

Matthew R. MUENSTER

Date: _____

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant : Graff et al.
Serial. No : 10/002,631 Examiner: To Be Assigned
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7/28/01

DECLARATION UNDER 37 C.F.R. §1.131

Professor Jonathon M. Graff residing at 3124 Milton Ave, Dallas, TX, 75205 and
510 Rochelle. NEN
75062 NEN
Dr. Matthew R. Muenster residing at ~~2014 Royal Oaks Drive~~, Irving, TX ~~75060~~ declare as follows:

1. We are the applicants of the above-identified patent application and coinventors of the subject matter described and claimed therein.
2. Prior to March 9, 2001, we completed the invention described and claimed in the subject application, in this country. The following set of facts demonstrate conception and reduction to practice of the subject matter of the present invention prior to March 9, 2001, the filing date of Tan et al. (US2002/012755A1).
3. Prior to March 9, 2001, Matthew R. Muenster prepared a vector comprising an ampicillin resistance (AmpR) gene with the native signal sequence deleted as a backbone vector for cloning the vector of the present invention. A copy of the relevant notebook data pages is attached as Exhibit A. The dates from Exhibit A are redacted.
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selection media. Expression of a cDNA fragment encoding a protein comprising a signal sequence confers survival of the host cell on selection media. A copy of the relevant notebook data pages is attached as Exhibit B. The dates from Exhibit B are redacted.

5. Prior to March 9, 2001, Matthew R. Muenster confirmed the sequence of the vector comprising the leptin gene cloned upstream of the AmpR gene. A copy of the relevant notebook data pages is attached as Exhibit C. The dates from Exhibit C are redacted.

6. Each of the dates redacted from Exhibits A-C is prior to March 9, 2001. Furthermore, each of the dates redacted from Exhibits A-C is prior to March 30, 2001.

7. We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jonathon M. GRAFF

Date: _____

x Matthew R. Muenster
Matthew R. MUENSTER

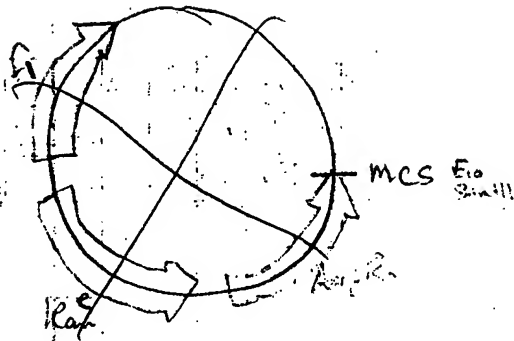
Date: x 7/9/03

Cloning of a bacterial vector to generate use for secreted protein screening.

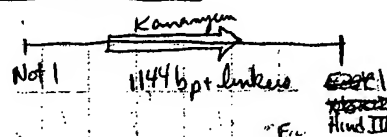
Strategy

1) PCR 2 distinct fragments from the original vector, which when ligated will give the desired vector

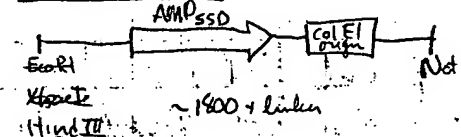
Fragment 1 will contain the kanamycin resistance gene and the first part of the gene.
Fragment 2 will contain the amp gene (w/o SS) and the ColEI origin.



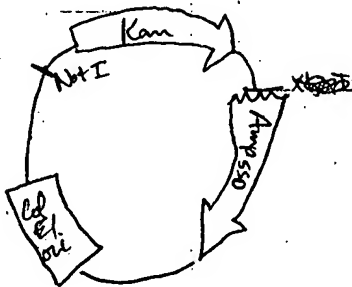
Fragment 1



Fragment 2



2) Once Ligate the two fragments to generate:



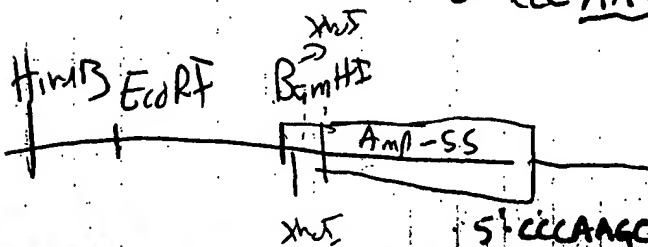
Now clone an ^{deep} from that introduces
EcoRI, ~~XbaI~~, ~~XbaI~~, ~~SpeI~~ sites into the HindIII
site. Clone the ~~XbaI~~, ~~XbaI~~

Kana 5' primer

5'-CCC ~~AGCT~~ TCA GGG CGCA A GGG CTGC -3'

Kana 3' primer

5'-CCC AA GCTT ACTCTT CCTTTT CAA TTC AG



Size: 1162 bp

Amp 5' primer

5'-CCCAAGCTT GATTTC ~~CA~~ CACCAGAAACGC TGG TG

Amp 3' primer

5'-CCC AAGCTT ATGTGA GCAAAA GGCC AGC -3'

Size - 1642 bp

5' - Seq Primer

5'-CCTTCTATCGCTTCTTG-3'

5'-CGCCGCTCCC GATTTC GCA GCGC

tal 182 gN

used 5 μ l of this to 100 μ l "old" comp cells and plated the entire amount onto 2 plates that JMP + I made. I got 0.1 colony total and grew this up. cells are shown above.

Either. Bgl II did not cut
or there is no Bgl II site
as the sequence says.

[illegible]

2500 11/25/50

1927 1928 1929 1930 1931 1932 1933 1934 1935 1936 1937 1938 1939 1940 1941 1942 1943 1944 1945 1946 1947 1948 1949 1950 1951 1952 1953 1954 1955 1956 1957 1958 1959 1960 1961 1962 1963 1964 1965 1966 1967 1968 1969 1970 1971 1972 1973 1974 1975 1976 1977 1978 1979 1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2

'E- JPA JJP AAAADP ADT TTA TTJPA JJJ

gd Spd - gnd

[illegible]

Prepare to PCR fragments for use in cloning the bacterial secretion screen vector.

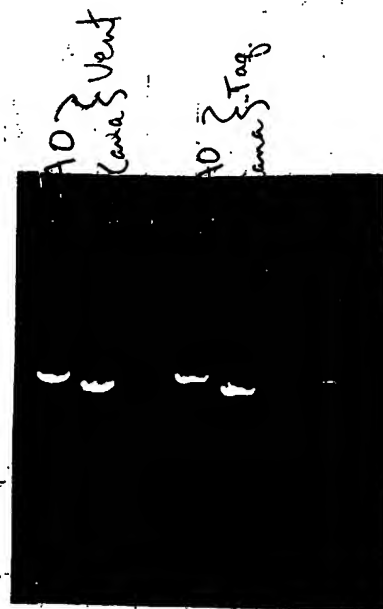
Set up the following Rxn's.

Pfu
 10 μ l Pfu Buffer
 4 μ l dNTP's
 1 μ l template (100 ng)
 5 μ l primer mix (50 ng/ μ l each)
 1 μ l Pfu Turbo
 79 μ l water
100 μ l total.

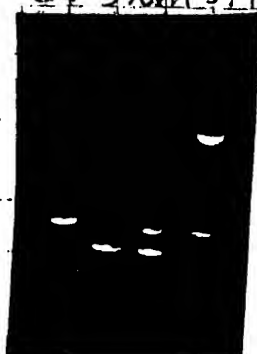
Tag

Tag
 2.5 μ l Tag Buffer
 0.8 μ l $MgCl_2$
 0.2 μ l template
 1 μ l primer mix
 0.25 μ l Tag
 1 μ l dNTP's
 19.25 μ l water
25 μ l total

Rxn 1: - AD - Pfu.
 2: - Kan - Pfu.
 3: + Cont - Pfu.
 4: AD - Tag
 5: Kan - Tag.
 6: + Cont - Tag.



PCR cleaned the AD-Vent, Kan-Vent, and a Mix of the Tag Fragments. Eluted to 50 μ l EB. I digested 25 μ l of each eluate by adding 6 μ l React II, 2 μ l Hind III and 2 μ l Xba I. 3 to 1 hr. Also did test digestions to ensure that the enzymes worked. That is shown to the right. PCR cleaned the 3 rxn's. Ran 2 μ l of each out on a gel.



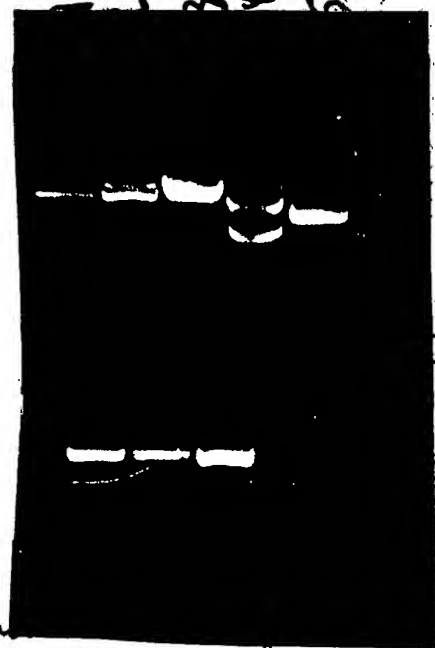
TA clone the Vent fragments in the following reactions.

2.

AO-Vent- PCR A-tagged		Kana-Vent- PCR A-tagged		(P) Control	- Control
Van Buffer	5 μ l	5 μ l	5 μ l	5 μ l	5 μ l
DNA	1 μ l	1 μ l	2 μ l (Control insert)	2 μ l	0 μ l
M-T-Easy	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
DNA Ligase	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
Water	2 μ l	2 μ l	1 μ l	3 μ l	3 μ l
	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l

Plated all 10 ligations + pCRII + EB3 in ^{pCR2.1}pCRII to different plates.

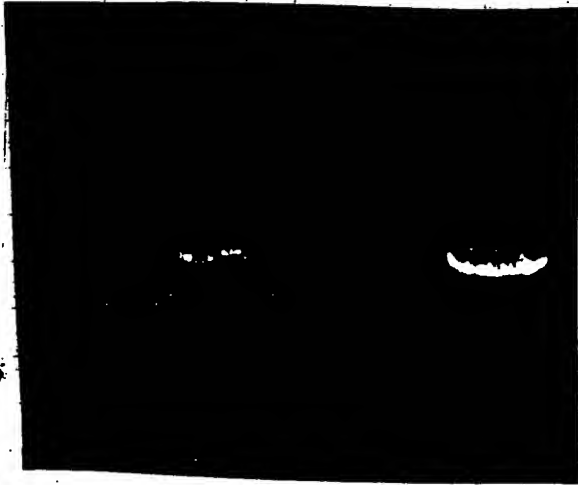
	Amp	Kana	Kana+Amp	Hemk Kana
Vent - lig	—	0	0	—
Vent + lig	—	1	0	—
Tag - lig	—	0	0	—
Tag + lig	—	0	0	—
SIDTP - lig	2	—	—	—
SIDTP + lig	~350	—	—	—
TA-AO	~300	—	—	—
TA-Kana	~500	—	—	—
TA+cont	~300	—	—	—
TA-cont	~40	—	—	—
pCRII xform	lawn of colonies	~200	~60	~35
EB3	lawn of colonies	~300	~250	~60
pCRII glycol	—	lawn of colonies	lawn of colonies	—



Picked 5 colonies from the TA-Kana-fragment plate and plated. Grew the AO's in LB+Amp and grew the Kana-clones in LB+Kana. Only 3 of the LB+Kana's grew. All five of the AO's grew. I miniprep'd these and digested them w/ Hind III. All 42 appeared to be correct for the AO and all three clones of the Kana fragment K1, K2, K3 were correct. I cut out the bands, cleaned them, and setup ligations w/ them.

Did an $EcoRI$, $BamHI$ digestion of $pCRII$ to make a vector in which to clone my $XcmI$ digests so as to convert $pCRII$ to a "homemade" TA cloning vector.

~~$BamHI$ linear~~
 $pCRII$
 $BamHI$ dig
 $EcoRI$ dig
 Eco/Bam dig



Either ^① $EcoRI$ did not cut
or ^② there is no $EcoRI$ site
in my clone.

Hypothesis 2 is consistent with
the digest I did on 7/24 where
I did an Eco/Bgl double digestion
and concluded the Bgl did not
work or its site was missing.

I need to sequence through
the MCS of my clone to see
what I got in the MCS.

apping the new construct w/ Rsa I.

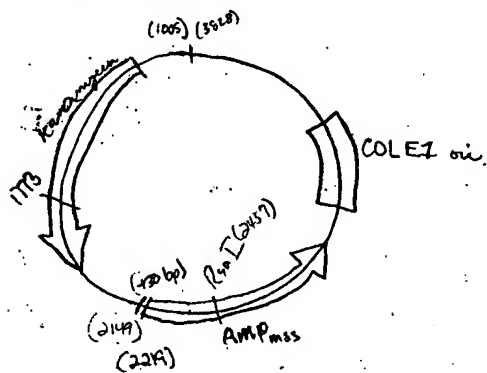
Rsa I digests the parent plasmid @ 285 bp, 1773 bp, 2457 bp.
This yields fragments of 684, 1488 bp, & 1728 bp.

My new clone will include bases 1005-2149,
2219-3828.

There are 2 Rsa Sites in my new clone.

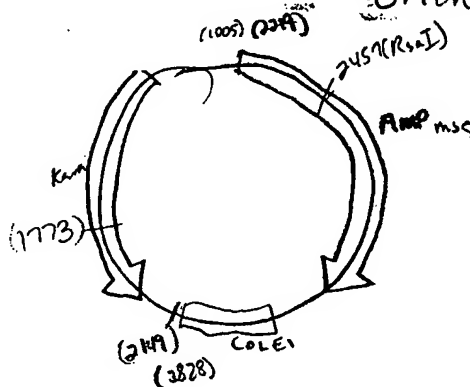
2 possible clones (orientations)

Orientation 1



Rsa I yields 644 bp & 2140 bp.

Orientation 2.



Rsa I yields 1030 bp & 1747 bp.

~~Set~~ Setup the following ligation using the generated fragments:

	K1	K2	K3	A's	K's
2x Ligase Buffer	5	5	5	5	5
T4 Ligase	1	1	1	1	1
frag K1	1.0	0	0	0	0.33 0.33
frag K2	0	1.0	0	0	0.33
frag K3	0	0	1.0	0	0.33
frag E A1	0.25	0.25	0.25	0.5	0
frag A2	0.25	0.25	0.25	0.5	0
water	2.5	2.5	2.5	3	3
	10	10	10	10	10

Ligated @ RT for 1 hr.

X⁻formed 20ul ONE SHOT'S from Herk Lab + 5ul of each ligation
5 min, - 1 min, 2 min, 40 min

Plated half of each tube to LB + Kana; and LB + Kana + Amp plates.
Colony counts are shown below:

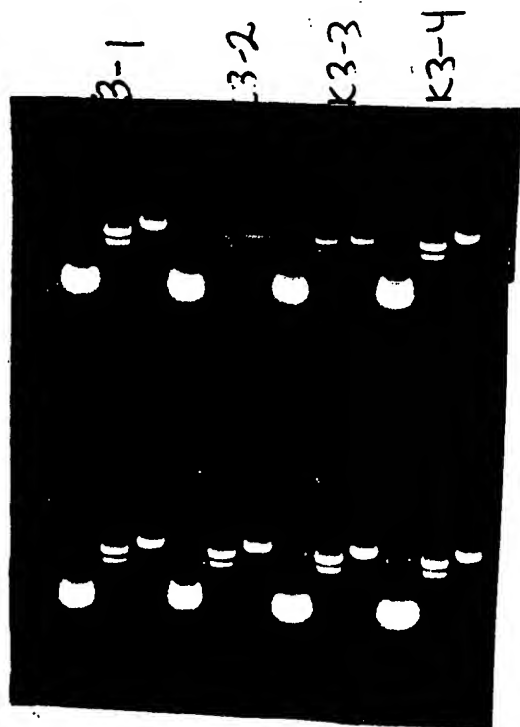
	LB + Kana	LB + K + Amp
K1	13	0
K2	10	1
K3	32	0
A's	0	0
K's	0	0

RsaI Digests

[illegible]

... 1 of TSV @ batopni

2nd shot 1/2 min. 1 min. 2 min. 40 min. at shot hole of first hole.



K3-1,4,5,6,7,8 are ~~the~~ correct in orientation 1.

$K_{3,2,3}$ are correct
and in orientation 2.

I will grow up K3, 1-4.
and midisep these
~~the~~

Student's Name

Subject

Date

Instructor's Name

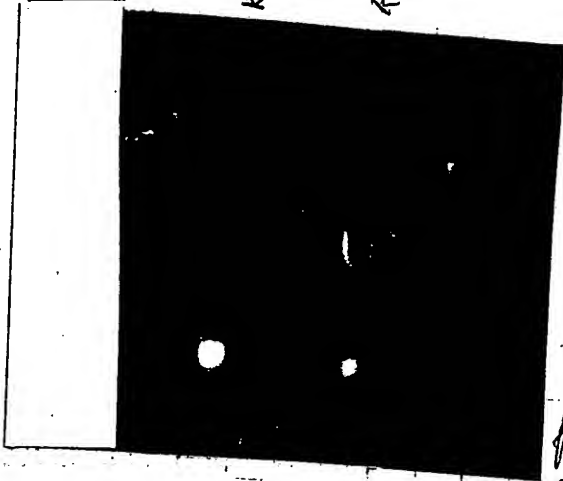
19

K3-1 UC
BamH1
EcoR1

K3-2 UC
Bam
Eco.

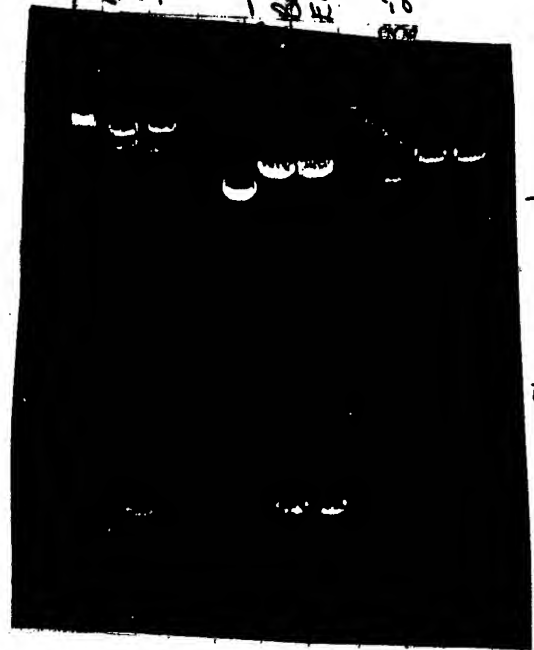
K3-3

K3-4



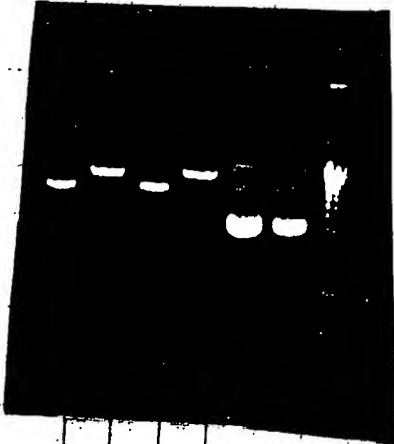
These
look
screamed
up and
different
from the
previous dig
So I will
do the midpreps
and repeat the
digestions

Unad
Bam
Eco.
-1 mid prep
BamH1
EcoR1



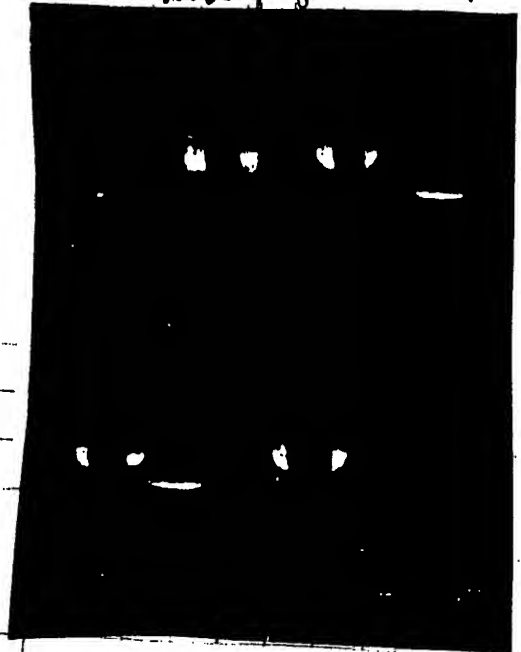
These
look
good
as to
the E.
and
Bam
site
are a
and
give
line
pro
the
correct
size

LEP2-Invariant
LEP2-Invariant BamH1



H1
Lam
p2,
PPA126
do

K3-1 to 4 dig w/ EcoR Bam
and fragments cut from gel.



Ordered primers for In-frame PCR to the amp construct

Ligations to Bacterial Vector

	<u>K</u>	<u>A</u>
	-200	-2000
pCMT	1	0
1-ins-lig	46	① - Pick
3-ins-lig	0	0
3-ins + lig	0	0
1 XGD	52	0
1-XGD	44	① - Pick.
3 XGD	413	0
3-XGD	17	0
1-T-LPL	60	0
1-V-LPL	~140	④ - Pick. → Pick 10
1-T-Lep	66	①
1-V-Lep	85	0 → Pick 10
1-T-PPAR α	49	7
1-V-PPAR α	~60	2 → Pick 10
3-T-LPL	0	0
3-V-LPL	0	0
3-T-Lep2	1	0
3-V-Lep2	1	① - Pick.
3-T-PPAR α	0	41
3-V-PPAR α	1	16

Screen 4 gene specific and one ^{vector specific} non-specific
Kana 5' + gene 3'

Controls Kana 5' + Kana 3'

2.5 ml PCR Buffer
0.8 M MgCl₂
1 ml dNTP's
1 ml primers
0.25 ml Tag.
19.5 ml water
25 ml

Picked colonies to LB + Kana. All the clones from the Kana plates grew but only the 1-T-Lep and the 1-XGD colonies from the amp plates grew.

Minipreped these and digested w/ Eco/Bam



Both clones contain inserts and these were sequenced in the UTSW lab.

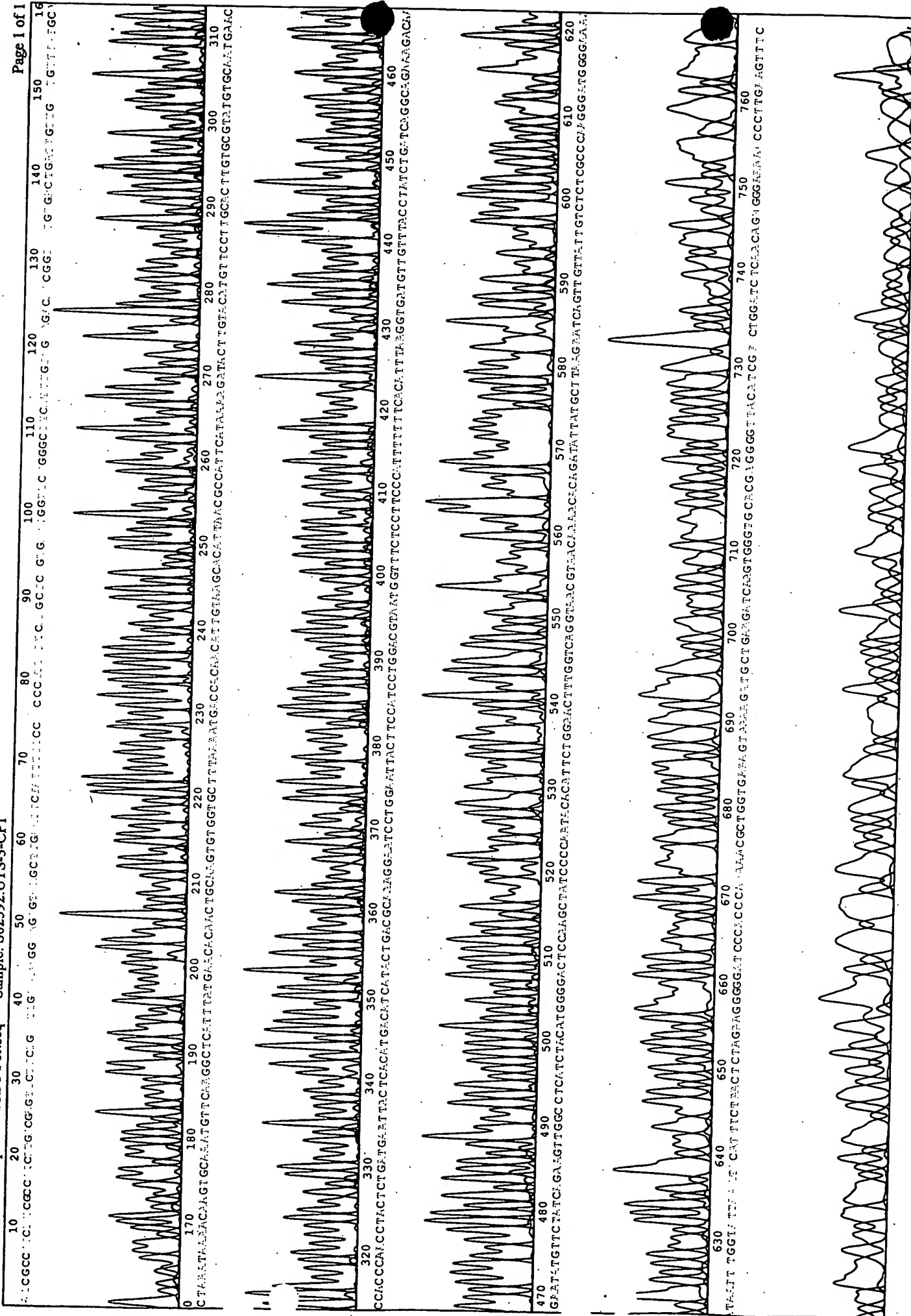
[illegible][illegible]

GGTAAAGATCCCTTGAGAGTTTCGGCCCCGAGACCGTTTCCCAATGATGAGGCCTTTTAAAGTTCTGCTATGTGGCGCGTTTTCCTGATTCACCGCGGGGCAAGCGACTCGCGGTTCGCGGTACACTATTCCTCAGAATGACTTGGTGGAG-

70
TCTCCCGTCACAGAAAGCATCTTAGCGGTGCGATGGCACTGGCAGTTAAGA GAAATTATGCCA GTGCTGCCATAACCATGAGTGTTTACC ACT GGCGCCACACTTACT TCTGCACACGATCGGAGG GCCGGAGGAGC TATCCCGCTTTTTTGCACACTTG

480 490 500 510 520 530 540 550 560 570 580 590 600 610 620

630 640 650 660 670 680 690 700 710 720 730 740 750 760
GGGATCTGTACTCGCCTTGTCGGTGGGACCGGAGCTGAAATGAAGCTACCACCGCGGTGACCCACGATGCCGTGCCTGGCACTACATTACCTGCTCTCCGGAA



Leptin fragment is exactly as I cloned it. So it appears good.
 The XGD fragment contains one ORF in frame w/ the β -lactamase gene.
 It codes for the following peptide.

Encoded by XGD insert.

MGKIILLNTFLTLEWGSHPETLVKVKDAEDQLGA
 Amp

euk network

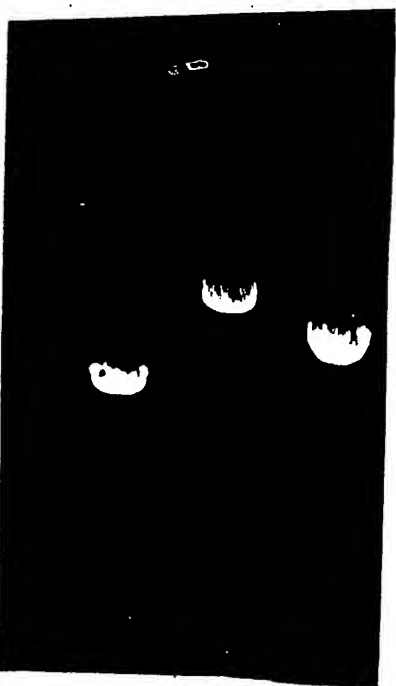
Is the sequence a signal peptide?

Feature	Position	Value	Cutoff	Conclusion
max. C	21	0.325	0.37	NO
max. Y	21	0.474	0.34	YES
max. S	13	0.934	0.88	YES
mean S	1-20	0.752	0.48	YES

Most likely cleavage site between pos. 20 and 21: SHP-ET

is this does potentially encode a secreted peptide.

then did PCR again and TA cloned the fragments of LPL, PPAR δ , & Lep.
 as were then cut out and gelcleaned.



Vector
 Sizes
 and
 wrong
 But
 I proceeded
 anyway

